

SPOROGENESIS IN BACTERIA
A STUDY OF TECHNIQUES

by

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INTRODUCTION

The role of bacterial spores in the spoilage of foods and in broadcasting diseases in man and animals is well known. Spores not only cause tremendous economic losses but also create public and livestock health problems. The well known features of bacterial spores have made them of unusual biological and practical interest. In the last few years laboratories all over the world have been actively engaged on particular aspects of the subjects and many excellent observations covering various aspects have appeared in the literature.

With few exceptions, endospores are formed by the bacteria belonging to two genera, viz. Bacillus and Clostridium. Recent studies have shown that, in general, spore formers follow a regular pattern of life-cycle, which sequence of events could be put forth as: vegetative cell; fore-spore; sporangium; endospore; germinating cell; out-growths; and finally completion of the cycle back to the vegetative form.

The present study was undertaken to study transition of the cell from the vegetative phase to the fore-spore and sporangium forms. Several have suggested that sporulation was the result of a mechanism or a factor, which has been commonly termed as the "triggering" factor. When once the cell triggers to form resting spores, a cycle of events is initiated which eventually leads to the formation of endospores. The "triggering" causes the growing cell to suspend its growth and multiplication in the vegetative form and initiates the formation of protective, resting form, the endospore. On close examination, the signs of spore initiation in a cell could be first detected by the appearance of a forespore in the vegetative cell. Eventually, for such type of study, development of suitable techniques by which

the transition of the cell form, from one stage to the other could be closely studied appeared very essential. A general review of the literature revealed that only few apparatuses and techniques have been utilized for such type of studies. Some workers have tried the use of chemostat but with little success. Therefore, in the present study an attempt has been made to use or develop some newer techniques which could be of help in such type of studies. Besides the shaking machine and the Warburg apparatus, light and electron microscopes, millipore-filter and gradient plate techniques, as well as methods of chemical analysis of spore components, were used with fruitful results. Very precisely, the main intention of the work has been to examine one aspect of bacterial spores, namely, the transformation of a vegetative cell to a spore cell -- the actual metamorphosis -- as well as the effects of environment on it, and development of some new techniques to suit these studies.

REVIEW OF LITERATURE

Sporogenesis

The present study was based on the background of several outstanding reviews on the formation of endospores (Cook, 1932; Knaysi, 1948; Lewis, 1934; and Robinow, 1960). The rod-shaped bacteria of the two genera Bacillus and Clostridium are the best known spore formers. At the end of their period of growth, a new cell or spore arises from their cytoplasm. The circumstances which induce bacteria to form spores and the changes which they undergo during this process was taken as the subject matter of this study.

From a review of the literature it appeared that sporulation by aerobic members of genus Bacillus has received more and extensive attention than the

members of the genus Clostridium. Based upon some early studies, several hypotheses were formed regarding the process of sporulation. Out of these, three of the hypotheses stand out prominently.

The first one of these suggested that the endospore was formed by the growth of a specialized granule in the bacterial cytoplasm (Koch, 1876; de Bary, 1887; and Ward, 1895; in Bayne-Jones and Petrilli, 1933). The second, that the endospore was formed by the fusion of a number of granules, some of which may be nuclear material (Babes, 1889; Dobell, 1908, 1909 and 1911; Ernst, 1888-89, and Schaudinn, 1902; in Bayne-Jones and Petrilli, 1933). The third hypothesis stated that the endospore was formed by gradual condensation of the cell substance, possibly due to dehydration (Matzuachita, 1902; and von Darsányi, 1927 and 1930; in Bayne-Jones and Petrilli, 1933). Cook (1932) stated that de Bary's account of the growth of the spore from a granule could not be bettered. Knaysi (1946) suggested that possibly the difference between the three modes was due to a different state of the nuclear material.

Ordal (1957) at the Illinois Symposium on Spores emphasized that "Our knowledge about 'why and how' a vegetative cell sporulates was still very limited." He also brought forward the following four common hypotheses of the time. The first and the oldest of them was that of Behring (1889; in Ordal, 1957), "Sporulation is an intermediate stage in the normal development of the bacterial cell, which may be partially or completely inhibited by some partial physiological damage short of total prevention of growth." The second was Knaysi's (1948; in Ordal, 1957) "Endospore are formed by healthy cells facing starvation." The third was of Foster and Heiligman (1940; in Ordal, 1957) "Sporulation is a sequence of integrated biochemical reactions

which are independent of vegetative growth and may be interrupted at certain susceptible stages." The fourth of these was that of Schmidt (1955; in Ordal, 1957) "Sporulation is a function both of environment and of cellular factors determining the reaction of the cell to a given environment." Very befittingly, the above referred author also summarized an acceptable concept suggesting that, "Sporulation is a normal metabolic process which will occur within the bacterial cell when:

- i) the cell is of a sporogenous type;
- ii) the cell acquires a proper physiological condition; and
- iii) the cell is surrounded by the proper environment."

All the above mentioned hypotheses and observations have gone a long way to create interest in spore research. During the last decade the subject has received much more attention and has been approached from many different angles. Based on these, Powell and Hunter (1953) stated that "... it is clear that sporulation involves concentration and possible de novo synthesis of cell material ..." However, this suggestion has since been modified and extended greatly by Foster and Perry (1954) and Foster (1956).

The "triggering" Mechanism

It has been suggested by Bayne-Jones and Petrilli (1933), Knaysl (1948) and Hardwick and Foster (1952) that the formation of bacterial endospore was "triggered" in some fashion and that this "triggering" might be the result of some reaction or a series of reactions in the vegetative cell which caused it to suspend the growth, division and multiplication and to initiate the formation of the spore. Bayne-Jones and Petrilli (1933) and Foster (1956) stated that this "triggering" would result in an irreversible commitment to sporulation.

Environmental Factors

Oxygen Requirement. Among the effects of various environmental factors on sporulation, the requirement of oxygen has been studied in greater detail. Knaysi (1945 and 1948) has forcefully stated that "... the necessity of oxygen for the quick and efficient formation of large number of endospores is one of the incontrovertible facts of bacteriology." Roth et al. (1955) stated that lower oxygen rates markedly reduced the percentage of sporulation. Earlier, Burnstetter and Magoon (1932) had reported that "... biochemical transformation in the cell leading to spore formation requires oxygen."

Effect of Shaking. Foster and Heiligman (1949) reported the use of a continuous shaking machine for maintaining homogenous and uniform physiological conditions in their cultures. Hardwick and Foster (1952) reported that although short periods of anaerobic culturing did not irreversibly destroy the sporogenic properties of the cells, they suggested that constant aerobic environment was to be strongly desired. Curran and Evans (1954) reported that shaking increased the numbers of spores in their experiments. According to Foster (1956), mechanical shaking for homogeneity was almost universal at the time of his report and suggested that limitation of aeration may occur without this precaution.

Energy Requirement. From the literature, it is difficult to evaluate the effect of energy sources on sporulation per se because there was no clear differentiation between cell requirements for growth and for sporulation. There has been considerable speculation on the effect of glucose per se (Ordal, 1957). Hardwick and Foster (1952), using replacement culture techniques, presented evidence that glucose affects sporulation of B. mycoides.

Thus, the lack of information about the energy source in the literature acted as an initiative in designing the experiments of the present studies.

Requirement of Metal Ions. Presence of high concentration of calcium in the spores of various aerobic bacilli as compared to the vegetative cells were first reported by Curren et al. (1943). Vinter's (1956 and 1957; in Arima and Kobayashi, 1962) experiments suggested that a high calcium content was essential for formation of normal spores. Sugiyama (1951) reported that calcium deficiency leads to reduced spore formation and to a lowered heat resistance of the spores thus formed. Slepecky and Foster (1959) reported that although some calcium was essential for formation of spore, exceptionally high calcium content appeared not to be uniquely associated with the spores' refractility, or resistance to desiccation, phenol or ultraviolet irradiation. There was, however, clearly an involvement of this ion in the thermal resistance of spores. Their results of studies with a chemically defined medium and analysis for metal contents of spores showed that the content of individual metals in spores was flexible within a wide range and depended upon the relative concentration in the growth medium. Thus various cations in spore seem to be interchangeable to a certain extent.

Some recent studies including those of Kolodziej and Slepecky (1962) and Weed (1963) have shown a highly specific effect of copper on sporulation process over a relatively narrow concentration.

Biochemistry of Sporulation

Various workers have shown that vegetative growth and sporulation are distinct in their response to several agents (Hanson et al. 1963). They reported early (Hanson et al., 1961) that a citrate synthesizing system was

active during the sporulation cycle, not during logarithmic growth. The appearance of citrate forming activity paralleled the disappearance of acetate from the culture medium. Nakata (1962) reported that utilization of acetate corresponded to granulation of the cells. Hashimoto, Black and Gerhardt (1960) reported that cells are irreversibly committed to sporulation when granulation occurs. Hanson et al. (1963) reported that the beginning of morphological changes associated with sporulation occurs as the pH begins to rise. It has been shown that glucose is oxidized to acetic and pyruvic acids as well as CO_2 (Nakata and Halvorson, 1960). Acetic acid is oxidized to CO_2 and also converted to poly- β -hydroxybutyric acid during sporulation (Nakata, 1962). He suggested that the inability of the cells to oxidize acetate and the lack of oxygen demand in the latter stages of sporulation are interesting phenomena and deserve further explorations. Energy for the growth is provided by glucose oxidation (Doi, Halvorson, and Church, 1959; Blumenthal, 1961), whereas acetate oxidation provides energy required during the initial stages of the sporulation cycle (Hanson et al., 1963).

Dipicolinic Acid

The one chemical compound that has aroused greatest interest in spore biology is the dipicolinic acid. Its unique nature is dependent upon the following characters (Foster, 1959):

- i) dipicolinic acid is present in all bacterial spores which have been examined up to the present time;
- ii) dipicolinic acid has not been reported to occur in any other place in the biological kingdom;
- iii) dipicolinic acid is found in extraordinary concentrations in bacterial spores; and
- iv) bacterial spores display extraordinary properties as compared to vegetative cells.

From these it is deduced that one or both the unique properties of the bacterial spore, namely, its negligible metabolism and its ability to survive environmental stresses which are lethal to vegetative cells, are attributable, in part at least, to dipicolinic acid.

Dipicolinic acid (DPA) was crystallized and identified from the slime of "Natto" (Japanese food prepared from steamed soybeans) inoculated with Bacillus natto (B. subtilis) by Udo (1936; in Arima and Kobayashi, 1962). It was then reported as a strong inhibitor for the growth of microorganisms. Later, Powell and Strange (1953) found that the calcium salt of DPA was released from spores of B. megaterium during germination.

Since the demonstration by Powell (1953) that bacterial spores contained dipicolinic acid, there has been much interest in its biological role. Several investigators have sought a correlation between the heat resistance of bacterial spores and their DPA content (Church and Halvorson, 1959; and Black et al., 1960). Doi and Halvorson (1961) presented evidence that DPA could be involved in an electron transport system. Riemann and Ordal (1961) reported that calcium dipicolinate (CaDPA) caused the germination of spores of many species of the genera Bacillus and Clostridium.

Curran, Burnstetter, and Myers (1943) and others have demonstrated that spores contain more cations than do vegetative cells. Riemann (1961) proposed that CaDPA could disrupt a normal stereostructure. Vinter (1961) stated that bacterial spore coats were rich in cysteine. Lee and Ordal (1963) suggested that perhaps, like ion-exchange resin, dormant spores could provide an insoluble matrix for the chelation of CaDPA and other insoluble materials which would make them "insoluble" in water.

Other studies have indicated that the dormant spore is anhydrous and yet permeable to water. On the basis of their refractive index measurements Ross and Billing (1957) concluded that dormant spores were anhydrous. According to the data presented by Lee and Ordal (1963) dormant spores are impermeable to the extent that they release only a limited amount of soluble constituents.

As a result of several studies, it became obvious that the DPA content of spores varied between 5 to 15% of the spore dry weight (Arima and Kobayashi, 1962). Usually, DPA does not exist in the vegetative cells.

The role of DPA in spores is not quite clear and many hypotheses have been suggested for the same, such as calcium retention in spores (Vinter, 1956; in Arima and Kobayashi, 1962), contribution to heat resistance of spores (Church and Halvorson, 1959; Levinson *et al.*, 1961), and stimulation of reduced diphosphopyridine nucleotide oxidase activity (Doi and Halvorson, 1961). Keynan and Halvorson (1962) suggested that DPA itself appears to have at least two functions: it drives the L-alanine dehydrogenase reaction, and it induces a "metabolically dependent" germination, independent of the L-alanine trigger mechanism.

Very little is known of the precursors of the dipicolinic acid (pyridine-2,6-dicarboxylic acid) in bacterial spores. The surprisingly high rate of conversion of so many normal metabolites to DPA is consistent with the hypotheses of a *de novo* synthesis of endotrophic spores from pre-existing components of the progenitor vegetative cells (Foster, 1956).

Many possible roles of DPA in the dormancy of spores have been suggested: (a) that it forms a part of the spore cortex (Mayall and Robinow, 1957; Foster, 1959; Rode and Foster, 1960), (b) that dipicolinic acid - calcium-protein complex stabilizes the spore against physical agents (Powell, 1953;

Young, 1959), (c) that DPA is a factor in heat resistant (Church and Halvorson, 1959) and X-ray irradiation resistance (Woese, 1959) and (d) that it is an activator of spore enzyme during germination by chelate mechanism (Harrell, Doi and Halvorson, 1957).

Slepecky and Foster (1959) found that calcium, manganese, and zinc formed chelation complexes with DPA but iron, potassium, sodium and magnesium did not. They also demonstrated that calcium and zinc effectively "compete" for uptake by spores. In addition, specific metals are capable of controlling two characteristic spore properties, DPA level and heat resistance (Slepecky and Foster, 1959; Black et al., 1960; and Pelcher and Ordal, 1961).

A number of independent lines of experimentations indicate that DPA is superficially located, probably as a shell external to vital core of the spore and internal to an outer protective coat (Foster, 1959). The striking refractility of the spore (Ross and Billing, 1957) is the most convincing evidence of its anhydrous state. Thus, the essence of sporulation can, therefore, be looked upon as a mechanism for, first, producing an anhydrous cell and, second, maintaining the anhydrous state (Foster, 1959).

MATERIALS AND METHODS

The main objective of the study demanded considerations of three basic requirements for the studies:

- 1) application or development of some suitable techniques which could help in the study of the transitions of the cell forms from one to the other;
- 11) some environmental conditions which could bring about early sporulation, or could delay the "triggering" mechanism under

controlled conditions; and

- iii) a spore forming cell system which would behave in a predictable manner under normal and controlled conditions.

Growth Medium

On closer examination of the above mentioned three main requirements it was felt that the selection or choice of a suitable medium demanded first and foremost attention. In this it appeared wise to avoid complex organic media and to use a simple, aparticulate liquid medium which would permit frequent removal of representative samples for observations during growth and sporulation and would normally support vegetative cell multiplication as well as sporulation.

Several workers have suggested and used synthetic medium in studies with aerobic bacilli (Foster and Helligman, 1949; William and Harper, 1951 and Slepecky and Foster, 1959). Others have pointed out the necessity of the presence of certain minerals in the environment of the sporulating cell. Manganese, calcium and requirements of certain other metals have been shown by Charney et al. (1951), Curran and Evans (1954), Slepecky and Foster (1959).

After due considerations of these reports as well as in an attempt to deduce an "ideal" system for the experiments to be undertaken, the final choice fell upon a medium suggested by Slepecky and Foster (1959). This was a chemically defined synthetic medium, which not only lent easy alterations in its composition during the course of experiments, but also eliminated the anti-sporulating factors like lipid or lipid-like materials usually present in organic media, reported by Foster et al. (1950) and Hardwick et al. (1951).

The medium suggested by Slepecky and Foster (1959) had the following composition:

Sucrose	1.0 g
KH_2PO_4	5.0 g
$(\text{NH}_4)_2\text{HPO}_4$	1.0 g
MgSO_4	0.2 g
NaCl	1.0 g
CaCl_2	5 mg
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	7 mg
ZnSO_4	10 mg
FeSO_4	10 mg
D. Water	1 L

A closer examination of this medium showed that its phosphate buffer composition required adjustments of the pH to 6.8 by addition of NaOH. This was avoided by using K_2HPO_4 in a required ratio. Thus, the final modified medium, which for the sake of convenience of expression in this work was designated as the "Synthetic Sucrose Medium" (SSM). The procedure used for making this medium involved several steps as outlined below.

1. Sucrose and sodium chloride, in the amounts indicated, were dissolved in 599 ml of distilled water. In order to get accurate concentration of magnesium sulfate, 2 g of this salt was dissolved in 10 ml of distilled water and 1 ml of this (containing 0.2 g) was added to the above solution.

2. A stock solution of mineral salts was made by adding 10 times the final concentrations of the salts to a liter of water. Care was taken to allow each of the ingredients to dissolve properly before addition of the next, to avoid chances of precipitation of the salts.

3. The phosphate buffer solution (PBS) was prepared in a separate container with the following:

$(\text{NH}_4)_2\text{PO}_4$	1 g
K_2HPO_4	3 g
KH_2PO_4	2 g
D. Water	100 ml

Each of the salts was added after the first one had fully dissolved. Determination of pH of this solution with a Coleman pH electrometer showed pH 6.9.

4. For the final preparation of the medium 1 ml of the mineral salt solution (2) was added to 60 ml of sucrose (1) solution and to which 19 ml of distilled water was added, distributed in growth vessels and autoclaved. In order to avoid precipitation of mineral salts in the presence of phosphate compound, phosphate buffer solution (3) was always autoclaved separately and added in 20 ml proportions to each flask with 80 ml of mineral-sucrose solution while it was still hot. Autoclaving for sterilization was carried out at 15 lb pressure for 40 to 45 minutes.

It may be mentioned here that during the course of experiments several alterations in the final composition of the SS medium were made to suit the requirements of the experiments. Thus, for the sake of simplifying the expressions the original medium was designated as the 1x, whereas when sucrose concentration was reduced to 1/2, 1/4 or so, or else increased, it was termed 3/4x, 1/2x, 1/4x, 1/8x, or 2x or so, respectively. When no sucrose was added at all it was called 0x.

Experimental Organism

Having made the final choice for a growth medium the next important need appeared to be the selection of a suitable aerobic sporulating cell system which would not only grow well and multiply in the SS medium selected, but would also form good spores. Thus, it seemed that an useful starting point would be to single out one organism of reasonably good size, easy to grow and which, under one set of conditions would reproducibly permit a

great majority of the cells to sporulate. With these ideas in mind some preliminary studies with 15 different aerobic spore forming organisms, available in the stock cultures were undertaken and after critical examinations and eliminations, a strain of Bacillus subtilis originally isolated from soil (Stock strain No. 28P2) was finally selected for use in the experiments. It was a motile, rod-shaped organism 2.0 to 3.0 μ long and 0.6 to 0.7 μ thick. Its vegetative form quite uniformly stained with 0.1% methylene blue. This had the advantage that not only the cells in various stages of sporulation took the stain, but also its spore could be observed with the method of staining used. Another big advantage with this organism was that when once its cells "triggered", it formed almost 100% spores in a short period of time.

Cultural Conditions

Stock cultures were maintained on nutrient agar slants and stored for future use in screw-capped tubes in the refrigerator at 4 to 5 C. For experiments the stock culture was first transferred to fresh nutrient agar slants. When fresh vegetative cells were required for sporulation studies in a fluid medium, 20 ml SSM in 50 ml Erlenmeyer flasks were inoculated with a few loops from a fresh culture on a slant and incubated for 12 to 14 hours. Repeated observations had shown that in SS medium maximum cell growth appeared between 14 to 16 hours after which the cells started showing a tendency to form spores. Thus, for almost all the studies where time factor for initiation was to be determined, the experimental growth flasks were inoculated with 12 to 14 hour old cultures.

In most of the experiments where SSM or an altered composition of SSM was used, 100 ml quantities of the medium were distributed in 250 ml flasks. The size of the inoculum depended upon the nature of the experiments. Incubation of cultures were carried out at 30 C (\pm 1 C). Samples of cells were usually obtained from a predetermined time onwards, at 2 hour intervals or more, depending upon the nature of the experiments. However, particular attentions were paid to obtain samples at the following 3 characteristic times during growth and sporulation:

- i) at the peak of the vegetative growth;
- ii) during the phase of initiation for forming spores; and
- iii) during the early phases of sporulation, i.e., the time when the formation of the forespore and sporangium started.

Growth was determined by visual observations of turbidity and by the measurements of the optical density of samples collected with a Bausch and Lomb Spectronic-20, using a wave length of 600 mu.

Shaking Machine. Since majority of the experiments were carried out with a fluid medium (SSM), culture flasks were incubated on a reciprocal shaker (Model S-3-53) by New Brunswick Scientific Co., New Brunswick, N. J., operating at 120 strokes per minute, with an oscillation of $1\frac{1}{2}$ inches.

Warburg Apparatus. Observations of the behavior of the cells in the absence of CO₂, as well as their manometrically measured oxygen uptake, during growth and multiplication were attempted using the common manometric techniques (Umbreit et al., 1957). Where absorption of the evolved CO₂ was desired, 0.2 ml of 10% KOH solution was put in the central wells of the flasks along with a filter paper strip. To have the same temperature of incubation the water bath was maintained at 30 C. A shaking rate of 120

oscillation per minute was maintained throughout the period of observation except at the time of resetting of the manometers' fluid levels or when the readings were recorded.

Gradient Plate Technique. This technique was used to run a few experiments using 2% (Difco) agar and SS medium. For these two kinds of media were prepared using 2% agar. In the first, sufficient agar was dissolved in measured volumes of SS medium to yield a synthetic sucrose - 2% agar medium and the other a plain 2% agar in distilled water. These were autoclaved in separate containers for 20 minutes at 15 lb pressure and cooled to 45 C before pouring the gradient plates. For each experiment 2 plates were poured with 20 ml SS-agar medium and allowed to set so as to yield a maximum gradient. When the agar was set each plate was arbitrarily divided into 3 zones by marking: High - Medium - Low according to the gradient obtained. One of these plates was over layered with 20 ml of plain 2% agar and the other with 10 ml of it and allowed to set. The control plates were also poured each with 30 ml of 1x agar and allowed to set without any gradient. These plates were prepared with adjusted times so that they could be used immediately after they were ready. Inoculation of these plates was carried out with 12 to 14 hour cultures grown on slants or in SS medium and incubated at 30 C. The behavior of the cells growing on these plates was examined by making slides at observable intervals from each of the zones on the marked plates as well as from those on the control plates.

Millipore-Filter Membrane. This procedure was used to study the effect of different concentrations of sucrose, the carbon source of energy in the SS medium under these altered conditions. This method, appears advantageous

in at least one respect over the gradient plate method. It permits the experimenter to vary conditions at any time, simply by transferring the membrane from the disc saturated with one medium to a disc with another medium. However, one difficulty which was experienced with this method was that cultures with heavier concentrations of cells could not be poured through these membranes. Therefore, samples had to be diluted in large volumes of sterilized fluid or distilled water before pouring over the membrane. For these experiments, required number of petri dishes, pads, cellulose filter membranes and the filtering apparatus were sterilized by autoclaving for 10 minutes at 15 lb pressure. Measured volumes of the fluid culture diluted with distilled water, were poured through the cellulose filter membranes (Millipore Filter Corp., Type HA grid; pore size, 0.45 μ). For obtaining negative pressure during filtration a tap connection attached to the filtration flask was used. Thus samples of cells, at the peak of their growth, were obtained on different filter membranes by allowing equal volumes of cell suspension through each. Each of these membranes were then transferred to labeled petri dishes containing different membrane-discs saturated with 1.2 ml each of 1x, 1/2x, 1/4x, and 1/8x SS medium, respectively, and incubated at 30 C.

Staining of slides was done with a 1 in 10 dilution of aqueous methylene blue for 2 minutes. Usually, rinsing of the slides after staining was avoided, especially when they were to be examined for the refractile stages, and were simply blotted between filter paper folds, dried and examined under oil immersion. This slight deviation of the classical procedure was found to greatly increase the ease of discrimination between various stages of the cell in the cycle for sporulation.

Electron Microscope

An attempt was made to obtain some electron microscopic pictures of the cells in the various stages of their sporulating cycle. For this, samples obtained at various time intervals from the growth vessels were processed in several ways before the final making of the grids. However, it was found that centrifugation and simple washing of cells in distilled water for 3 to 4 times was a good simple method for obtaining clean grids. Carbon grids obtained from M/S Bitter Root Specialty Company, Hamilton, Montana were used for this work. For making the grids a very small drop of the material was transferred to its surface and allowed to stay there for 4 to 5 minutes after which excess fluid was gently drawn off with the help of a thin Pasteur pipette and the grid was allowed to dry. Shadow casting, using platinum palladium was done on some of the grids but partially so in an attempt to possibly preserve the visibility of the internal structures as well as to slightly outline the external features of the cells.

Dipicolinic Acid Estimation

For estimation of the DPA content of sporulating cells, samples in 7 ml quantities were removed from the growth vessels at different intervals and stored below freezing point till processing. Processing involved repeated centrifugation of the samples, which was done at 5,000 r.p.m. for 25 to 30 minutes in anglehead "Servall" centrifuges. After the first centrifugation the media was decanted and then the cells were washed with 5 ml of 0.8% NaCl solution and centrifuged two more times. The supernatant after the last centrifugation was discarded and the cells were then resuspended in 5 ml of 80% ethanol and heated in the water bath for 2 hours at 56 C. On removal

from the water bath the samples were again centrifuged for 25 to 30 minutes at 5,000 r.p.m. and the supernatant obtained was used to determine the optical density in a Beckman Spectrophotometer (Model DU) using a wave length of 270 mu.

EXPERIMENTAL RESULTS

Effects of Energy Source

Before discussing the results obtained it would perhaps be appropriate to describe some of the stages the cells undergo during the cycle of sporulation, and the terms that have been used to describe them in this work. The first of these stages in the process involves the appearance of granules in the vegetative cells when stained. This stage is also accompanied or immediately followed by slight elongation of the cells which are easily apparent. The second stage is the formation of the forespore, and evidence of this is the sudden appearance of a partially refractile or a faint, elliptical portion at one end of the vegetative cell. The appearance of these cells has been used as the guide for initiation in this study. The third is the appearance of sporangium which refers to a vegetative cell containing the refractile, still immature spore about to be released; and finally the spore, when it is free of any vegetative counterpart. It then slowly decreases in width with little or no change in length, and assuming a more slender and smaller volume becomes the highly refractile, oval to round body, the endospore.

In the medium used in this study, cells from 18 to 20 hour old cultures contained granular areas, as the sites for spore formation. These cells proceed to complete the endospore formation even when transferred from the

old to a fresh medium or when fresh medium is added. Thus, in such instances, spore formation, once initiated continued to completion irrespective of new supplies of energy source. However, if fresh medium was added or else cells were transferred to a fresh medium, just prior to the appearance of such type of cells in the culture, vegetative multiplication continued.

Effect of Heavy and Light Inoculum. When two flasks each containing 100 ml of SS medium were inoculated with 2 ml and 0.2 ml of cells washed from a 12-hour growth on agar slant, it was found that the cells in the flask with the heavy inoculum had formed spores earlier than the cells in the flask with light inoculum. The results are presented in Table 1.

Table 1. Effect of heavy and light inoculum of a 12-hour culture on sporulation.

Inoculum	Time of incubation in hours							
	16		20		36		42	
	GT	%S	GT	%S	GT	%S	GT	%S
Heavy (2.0 ml)	++	0	+++	50	++++	100		
Light (0.2 ml)	+	0	++	0	+++	40	++++	100

GT = Growth turbidity.

%S = Percent of cells formed spores.

Effect of Varying the Concentrations of Energy Source. When five flasks with 100 ml each of 2x, 1x, 3/4x, 1/2x, and 0x were inoculated with a moderate size inoculum from a flask with a 14-hour culture in 1x SS medium, results indicated (Table 2) that lack of sufficient energy source caused early sporulation. When the experiment was repeated with a rather widely spaced variation in the concentrations of the energy source, the results were

similar to the previous experiment except that more information about the effect of decreased concentrations could be gathered (Table 3).

Effect of Used and Added Energy Source. Experiment was conducted with a 16-hour culture in lx medium from which cells were centrifuged and transferred to a fresh medium. A comparative study was made by using a sample of cells from a fresh culture obtained by centrifugation and added to the supernatant, or the used medium obtained after centrifugation of the 16-hour-old culture. The results showed that when fresh energy source was available the cells were not triggered to form spores but kept on growing and multiplying. However, if some cells had already initiated to form spores they did not revert and went on to complete the cycle.

Effects of NaHCO_3

When a 10% solution of sodium bicarbonate was added to the growth vessels with lx medium, in different quantities, it was found that addition of sodium bicarbonate had a definite effect on postponing the "triggering" time, although not preventing the sporulation altogether (Table 4). Whether this effect was due to carbonate-carbon dioxide relations or due to changes in the pH of the growth medium was not ascertained. However, Powell and Hunter (1955) had reported that compounds like bicarbonates or α -ketoglutarate furnished higher concentrations of CO_2 which acted as a stimulating factor.

Warburg Apparatus

A few experiments were run on the Warburg apparatus to determine the oxygen uptake during the phase of growth, initiation and sporulation, as well as to study the behavior of the cell in the absence of CO_2 . The failure

Table 2. Effect of different concentrations of energy source - sucrose - upon sporulation.

Sucrose concentration :	Hours at which samples were examined											
	13 : GI %S :	15 : GI %S :	17 : GI %S :	19 : GI %S :	21 : GI %S :	24 : GI %S :	28 : GI %S :	32 : GI %S :	36 : GI %S :	40 : GI %S :	44 : GI %S :	48 : GI %S :
0x	-	0	-	0	-	0	-	0	-	0	-	0
1/2x	++	0	+++	*	+++	*	+++	10	+++	15	++	80
3/4x	++	0	+++	0	+++	*	+++	5	+++	10	++	90
1x	+++	0	+++	0	+++	*	+++	2-3	+++	5	+++	99
2x	-	0	-	0	-	0	-	0	+++	0	+++	99

* = No spores formed but signs of initiation visible.

GI = Growth turbidity.

%S = Percent of the vegetative cells formed spores.

0 = No spores.

Table 3. Effect of widely spaced concentrations of energy source - sucrose - upon sporulation.

Sucrose concentration	Hours at which samples were examined											
	15	17	19	21	23	25	27	29	31	33	35	37
	GT	GS	GT	GS	GT	GS	GT	GS	GT	GS	GT	GS
1/8x	+	*	+	*	+	20	+	80	+	95	+	100
1/4x	+	0	++	*	++	2-3	++	50	++	90	++	100
1/2x	+	0	++	0	+++	*	+++	3-4	+++	80	+++	100
1x	++	0	++	0	+++	0	+++	*	+++	10	+++	95

* = No spores formed but signs of initiation visible.

GT = Growth turbidity.

GS = Percent of vegetative cells formed spores.

0 = No spores.

Table 4. Effects of varying amounts of added NaHCO_3 upon sporulation.

Quantity of 10% NaHCO_3 added in ml to flasks with 100 ml lx	Findings of the samples examined at:											
	(Hours post inoculation)											
	10	20	24	28	31	43						
	GT	GS	GT	GS	GT	GS	GT	GS	GT	GS	GT	GS
Control 0 ml	++	0	++	*	+++	10	+++	50	+++	80	+++	100
1/2 ml	++	0	++	*	+++	2	+++	40	+++	50	+++	100
3 ml	++	0	++	0	+++	0	+++	*	++++	20	++++	100
5 ml	++	0	++	0	+++	0	+++	0	++++	0	++++	100

GT = Growth turbidity.

GS = Percent of the vegetative cells formed into spores.

0 = No spores.

* = No spores formed but signs of initiation visible.

of electrical portions of the apparatus prevented adequate tests for conclusive evidence. However, a general observation that could be made was that cells in flasks with no CO_2 available not only failed to form good spores but in the stained preparations also looked beaded, rather translucent and of irregular sizes, more or less, indicating that the formation of cell walls as well as the synthesis of spores was being hindered in some way. These findings support the theories mentioned earlier that CO_2 is essential for good sporulation, though the suggestion would need further confirmation.

Estimation of Turbidity

Throughout the study attempts were made to measure the growth of the cells in two ways; by a comparative visual observation and by the use of a Spectronic-20 at a wave length of 600 mu. The determinations by visual observations were made by comparing the turbidities in the different flasks at particular phases of the growth in the various experiments, whereas for O. D. determinations representative samples from each flask were removed in 4 ml quantities and examined. However, with the spectrophotometer some difficulties were encountered which need elucidation. Because of the mucoid nature of the organism used in these experiments, the cells in the samples had a tendency to form clumps and to settle down to the bottom of the sample tubes. This made accurate reproducible turbidity readings difficult to obtain. However, in the latter stages of the study an effort was made to avoid this difficulty by collecting and examining the samples immediately after removing the flasks from the shaker, but the results plotted in Fig. 1 show

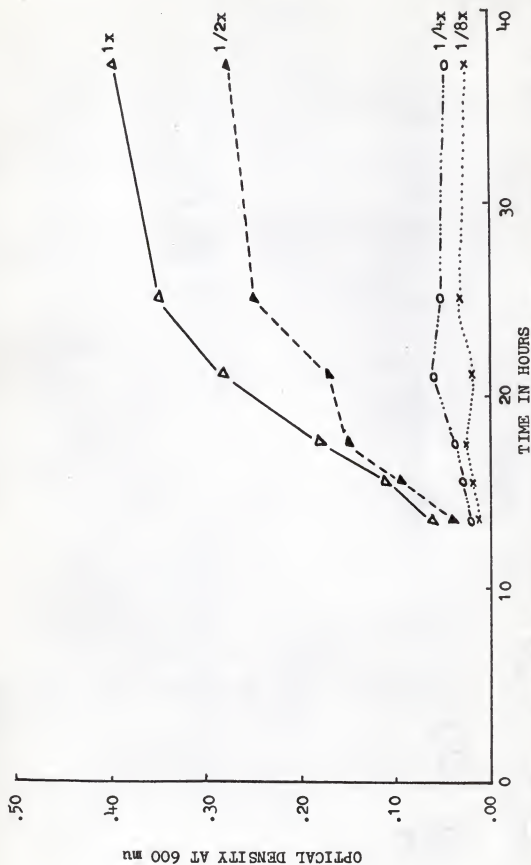


FIG. 1. Effect on growth turbidity of cells by different concentrations of energy source sucrose.

in spite of these precautions, some erratic observations could not be eliminated fully.

Millipore-filter Membrane

Other sustaining evidence that adequate energy source was essential for good growth and sporulation was obtained when cells of the same age obtained on different cellulose membranes were incubated on pads saturated with different concentrations of the SS medium. The results plotted in Fig. 2 indicate that cells sporulate early when there is lack of available energy source.

It should be mentioned that to obtain cell samples on the membranes, the cell suspension had to be sufficiently diluted or else filtration was not possible. Furthermore, only a very thin deposit of cells could be obtained on the membranes, making it difficult to obtain good smears for slide examinations without much disturbing the growth. However, the use of a magnifying lens proved to be of some help.

Gradient Plate Method

The result obtained by the application of the gradient plate technique clearly indicated that growth and sporulation was greatly dependent upon the amount of available energy source. However, it has to be appreciated that even with the maximum possible gradients obtainable in petri dishes, no clear demarcations could possibly be made between the high, medium and low zones. Still, from the slides made with care to obtain representative samples from each of the zones, it was found that at the time when initiation was quite evident in the low zone, cells in the medium and high zones were

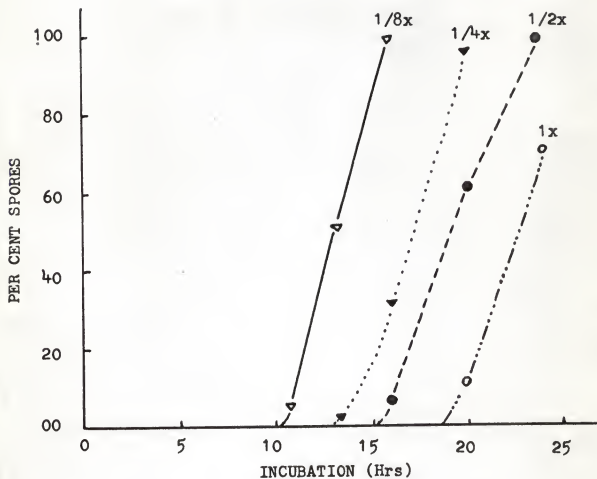


FIG. 2. Per cent spores formed on millipore-filter membranes when incubated with 1x, 1/2x, 1/4x, and 1/8x concentrations of energy source sucrose.

still multiplying. However, the medium zone showed earlier initiations as compared to the high zone. A few points of interest in these experiments were that a longer lag phase was observed on the plates that were overlaid with 20 ml plain agar as compared to the plate with 10 ml of overlay. Also the lag on plates with overlay was greater than on the control plates with no overlay. This perhaps was because of the physical type of distance between the substrate and the cells and perhaps also because the plates were poured immediately before use and thereby the two layers could not get time for diffusion. However, this situation was quite desirable under the conditions of the experiment.

Dipicolinic Acid Analysis

During the course of an experiment with different concentrations of the energy source sucrose, an attempt was made to estimate the amount of DPA synthesized by the sporulating cells. Though the result obtained can not be termed quite significant, yet it gives an idea that the amount of this compound is directly dependent upon the number of spores synthesized or that were in the process of being synthesized. Thus, from the results shown in Fig. 3 it would appear that in spite of the fact that the total number of cells in the various samples from 1x and 1/2x media were always greater, the amount of DPA synthesized by the cells of these flasks was consistently lower than by the cells in the 1/4x and 1/8x flasks. This fact becomes more evident when the results are compared with the observations that at the time when the 4th sample was collected, i.e., at 21 hours post inoculation of these flasks, only 30 to 50% of the cells in 1x and 1/2x flasks had formed forespores and sporangium and they were still to mature as

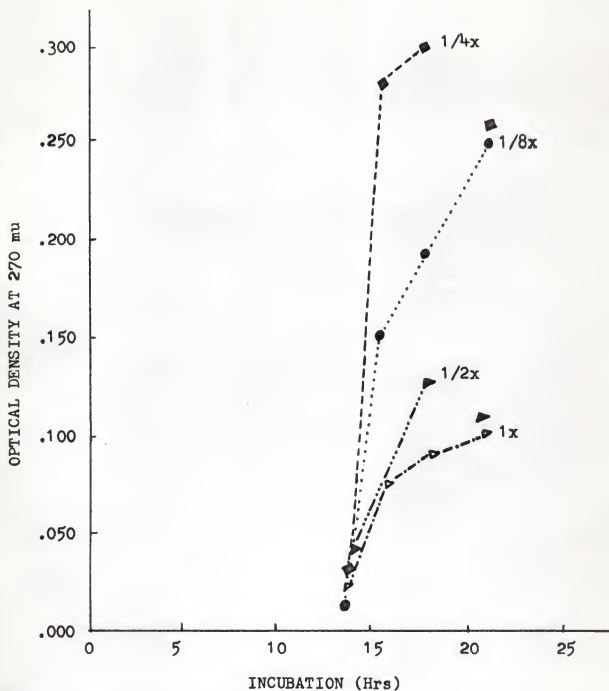


FIG. 3. Synthesis of dipicolinic acid by sporulating cells in different concentrations of sucrose.

spores; whereas in the 1/8x and 1/4x flasks on the other hand, 80% or more of the cells were rapidly maturing as spores. Thus, the results obtained signify that synthesis of DPA increases as the spores are formed and matured, which is a finding similar to that of Foster (1959) and of others, and that the method used in this study for the determination of this compound is quite workable.

Electron Microscope

A series of microphotographs taken with electron microscope present an appreciable view of the different stages of cells during the process of sporulation. Plates I to VI have been arranged to present the respective sequence of events as well as the lysis of cells which fail to sporulate. They go a long way to substantiate the results obtained in the various experiments.

DISCUSSION

The chief object of the present study was to examine one aspect of bacterial spores, namely, the transformation of a vegetative cell to a spore -- the actual metamorphosis. Several methods have been used by various workers to approach such and similar problems, and have been discussed in the Review of Literature.

Kinetic studies on vegetative growth and spore formation in the medium used in this study revealed that so long as the growth of the bacterial cell was in the logarithmic phase almost negligible degree if any, sporulation occurred. Sporulation commenced only after vegetative proliferation had virtually ceased. Similar observations have been reported by Knaysl (1946)

EXPLANATION OF PLATE I

Electron micrograph of a vegetative cell of Bacillus subtilis showing capsule and flagellum.

Shadowed with platinum palladium.
Magnification: approximately 61,500X.

PLATE I

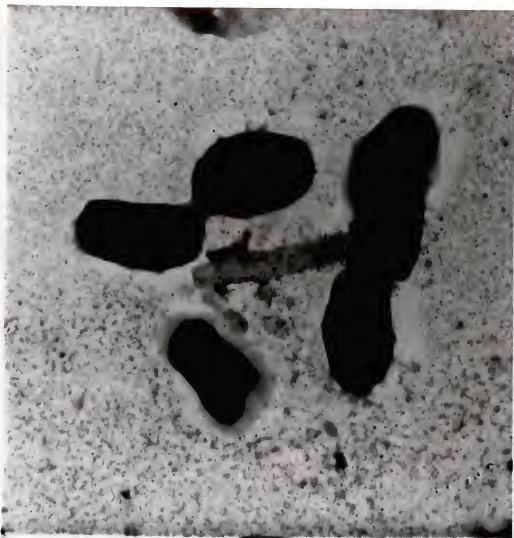


EXPLANATION OF PLATE II

Electron micrograph of four cells of Bacillus subtilis. One of the cells showing the newly formed, partially refractile forespore. The increase of the cell length during the process of sporulation is comparable with the two dividing cells in the field of normal size.

Magnification: approximately 19,000X.

PLATE II

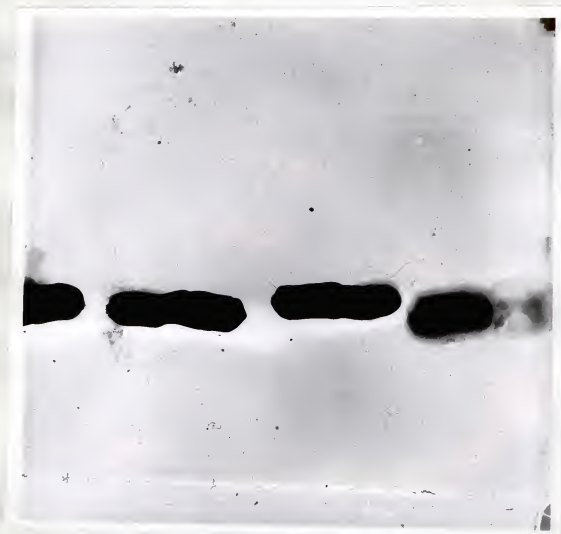


EXPLANATION OF PLATE III

Electron micrograph of three cells of Bacillus subtilis showing the sequence of appearance of forespore. The cell at the right hand end of the chain shows a stage which follows the forespore stage of Plate II. Other two cells have elongated and are in the process of forming forespore. Few flagella are also seen.

Shadowed with platinum palladium.
Magnification: approximately 16,500X.

PLATE III



EXPLANATION OF PLATE IV

Electron micrograph of one individual cell of Bacillus subtilis showing almost a fully formed spore but still attached to its vegetative counterpart. This is the stage referred to as the sporangium and follows the forespore stage. The gradual constriction is evident on comparisons between the vegetative and the refractile portions of Plates II, III, and IV, which leads to the release of the spore from the vegetative counterpart. Some of the inner structures of the forming spore are also seen as well as a flagellum in the vegetative portion.

Shadowed with platinum palledium.

Magnification: approximately 31,500X.

PLATE IV



EXPLANATION OF PLATE V

Electron micrograph of a fully formed spore free of its vegetative counterpart. Its external contour as well as some inner structures are seen. It is presumed that the refractile outer coat contains the dipicolinic acid.

Shadowed with platinum palladium.
Magnification: approximately 41,000X.

PLATE V

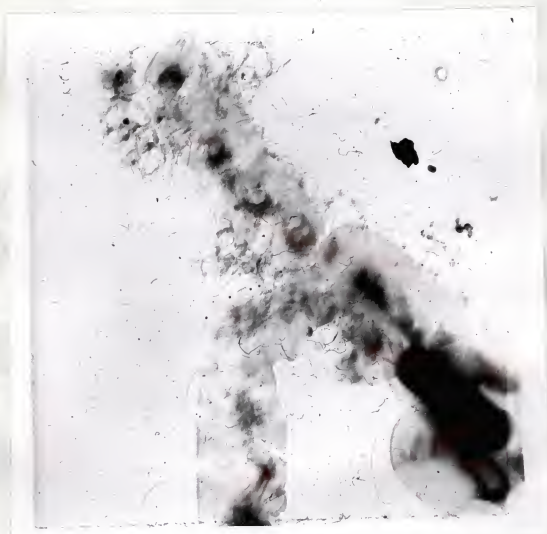


EXPLANATION OF PLATE VI

Electron micrograph of several cells of Bacillus subtilis. During the process of sporulation some of the cells fail to form spores and are lysed. The appearance of granules in one of the cells indicates that it had initiated to form spore but could not proceed further in the process, perhaps because of lack of energy source, and is disintegrating. Another cell on the top is also perhaps meeting the same fate.

Shadowed with platinum palladium.
Magnification: approximately 21,000X.

PLATE VI



and others with complex organic media, and Hardwick and Foster (1952) with synthetic medium.

It appeared that the phenomenon of sporulation is an autocatalytic process which is not necessarily geared to growth but which occurs near the end of the exponential growth period -- one vegetative cell producing one spore.

From the results obtained it appeared that there exists a very close relation between spore formation and the exhaustion of (nutrients) energy source essential for continued vegetative growth, perhaps as defensive mechanism. According to Oginsky and Umbreit (1954): "The process of sporulation does, indeed, result in a mechanism for defense; not, however, a bulwark hastily erected when the enemy is sighted, but a fortress that happens to be there when the enemy arrived."

The evidences obtained establish a somewhat unified picture of events occurring between the growth and sporulation in aerobic sporogenic bacteria in the synthetic sucrose medium. In this medium vegetative growth continued until the energy source was exhausted. Morphological changes associated with sporulation started appearing as the energy source diminished and growth ceased.

In stained preparations from growing cultures, the first premonitory signs of spore formation could be detected some two hours before the forespore (sporefield of some authors) could be observed. During this period of transition from vegetative form, several granules appeared in the cells which took stain. These granules were surrounded by a slightly refractile or clear zones. This was followed by slight increase in the cell length accompanied by a little widening of the cell at one end which gave the cell

more or less a club-shape appearance. The latter portion gradually elongated and thickened enough to show a clear demarcation between the two parts of the cell. This stage was followed by the gradual emergence into view of the smooth, transparent forespore at the club-shaped end of the cell, a positive sign of spore being formed. The transparent portion gradually became more and more refractile and while still attached to its vegetative counterpart, the forespore, slightly condensed in its size, to a stage referred to as the sporangium. In about 2 to 3 hours after the appearance of the forespore, the sporangium was released free of its vegetative counterpart and it then gradually ripened to a fully refractile endospore. It was also observed that during the process of ripening the spore which after immediate release from the vegetative counterpart had a slightly elongated contour and took the stain, gradually condensed to a smaller size which was then oval to round and took very little or no stain, denoting that the ripening was complete.

The synthetic medium used in this study proved advantageous in several ways. It made possible the shaking of cultures for providing a homogenous physiological environment for the growing cells as well as complete utilization of the medium which could not be possibly attained in still cultures. It supported the growth and sporulation of the cell system used as well as lending easy alterations in its composition for obtaining comparable results. It also, besides permitting frequent removal of samples for observations during growth and sporulation, facilitated the use of Warburg apparatus, gradient plates, millipore-filter as well as samplings for the chemical estimations of dipicolinic acid.

Although in the present work the effect of the carbon energy source, sucrose, was mainly studied, the medium used in the studies could very well be utilized in the studies of the effects of its nitrogen source or the mineral constituents. It could also support studies for DPA synthesis by sporulating cells and it would be perhaps of some interest to study whether heat-resistance of spores had any direct relation with the amount of DPA synthesized by the sporulating cells. The utilization of the different mineral constituents of the medium in the synthesis of DPA, aided perhaps by paper chromatography, appears to be another line of approach to the problem of sporulation demanding attention. Other areas of investigation may include uptake of metal components of the minimal synthetic medium and qualitative and quantitative differences in some basic constituents of cells harvested at characteristic times during growth and sporulation.

During the course of experiments where lower concentrations of sucrose were used, it was observed that depending upon the relative decrease of the concentrations, 100% cells were not able to form spores, and instead, some of the cells were being lysed. The number of cells that were lysed was directly related to the amount of energy source available in the substrate. It was also observed that in such situations the cells first lost their outer structure and at times appeared larger or like irregular yeast cells, rather translucent than the normal cells, and it is presumed that shortly after this their structure disintegrated. Nakata and Halvorson (1960) in their experiments with Bacillus cereus and glucose had obtained somewhat similar results.

The above observations suggested that cells when sporulating had an increased energy demand perhaps to sustain the endogenous changes leading

to the formation of spores. It also indicated that energy source is required not only for the growth but for sporulation too. Perhaps when the available energy source is below a critical level of requirement, cells failing to obtain their minimal requirements for forming spores, die and disintegrate. It could, therefore, be assumed that when the substrate contained a level of energy source sufficiently higher than the normal requirements of the cells in the culture, vegetative growth continued and triggering for sporulation was prevented. But when the level decreased to some critical point, the growing cells were "triggered" to form spores. Thus, the percentage of spores formed perhaps depended directly upon the level of the balance energy source that was still available in the substrate and the number of cells present there to sporulate. Therefore, if this level dropped below the minimum requirement for all the cells present, a proportionate number of them were unable to form spores. In this, perhaps nature's rule of survival of the fittest came into play and those unable to compete died and disintegrated. Thus, a basal level of energy source appears necessary for the maintenance of structure and physiological integrity.

Precisely, all these observations go to indicate that cells are very sensitive about their environment and perhaps there exists some enzyme regulating mechanism which directs as to which compound should be synthesized in a particular environmental condition and leads us to the domain of molecular biology.

The various techniques used in this study proved very helpful in obtaining comparable results and deserve trials in such and similar type of studies. However, with the millipore filter membrane, it was found that on being

incubated the filter pads saturated with the media showed a tendency to dry up, but this was prevented by placing the culture plates in a tray over a towel moistened with water. It should also be mentioned that the repeated washings and centrifugations of the cells for DPA estimation demanded great care at the time of removing the supernatant in order to avoid any chance of loss of cells in this procedure. The series of Plates (I to VI) obtained with the help of electron microscope have proved very helpful in correlating the story of the findings obtained during the various experiments. All in all, the work has yielded some interesting findings and has opened avenues for future investigations.

SUMMARY OF RESULTS AND CONCLUSIONS

An sparticulate liquid synthetic medium was developed and described. Its possible uses and advantages in its applications in varied experimental conditions, the ease of altering its composition as well as of obtaining samples for observations were demonstrated and discussed.

The effects of carbon source of energy upon growth and sporulation were studied and the findings discussed. It was found that energy source is required both for the growth and sporulation. Also a possible correlation between the amount of available energy source and sporulation were described and discussed. The synthesis and occurrence of dipicolinic acid in sporulating and vegetative cells were demonstrated and discussed.

Use and application of some new techniques, viz., gradient plate technique, millipore filter membrane technique as well as chemical estimation methods for DPA were shown and their advantages or otherwise were discussed.

Applications of these techniques in similar types of studies were discussed.

Different stages in the process of sporulation of an aerobic spore-forming cell system were described. With a series of electron microphotographs, different events following "triggering" for sporulation were demonstrated.

Further approaches for future studies were suggested and discussed.

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SPOROGENESIS IN BACTERIA
A STUDY OF TECHNIQUES

by

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The well known features of bacterial spores have made them of unusual biological and practical interest. Spores not only cause tremendous economic losses but also create public and livestock health problems and therefore, have engaged attention of a large number of workers all over the world. Many excellent observations covering various aspects of the problem have appeared in the literature during recent years.

The present study was undertaken with the following purposes:

- i) to examine one aspect of aerobic bacterial sporeformers, namely, the transition of a vegetative cell to a spore cell;
- ii) to study the effects of environment upon sporulation; and
- iii) to develop some new techniques suitable for such type of studies.

Thus, the circumstances which induce bacteria to form spores and the changes which they undergo during this process formed the main background for this study.

In order to study the effects of environment, an aparticulate synthetic medium was developed. After some preliminary studies with 15 different spore-forming organisms, a strain of Bacillus subtilis (stock strain No. 26P2) was selected for use in the experiments. This organism grew well and formed almost 100% spores in the medium used. Its good size and the fact that its newly formed spores could also be outlined with (0.1%) aqueous methylene blue, the stain used in the study, was very advantageous.

Attempt was made to develop some newer techniques which went a long way to help in the study. Besides the shaking machine and Warburg apparatus, millipore-filter and gradient plate techniques, methods of chemical analysis of spore components, as well as light and electron microscopes were used with

fruitful results. Advantages or otherwise in the applications of these techniques as well as of the medium developed, in such and similar type of studies, were discussed. The synthesis and occurrence of dipicolinic acid in sporulating cells was demonstrated and discussed.

Kinetic studies on growth and sporulation revealed that as long as the growth of cells was in the logarithmic phase, no sporulation occurred. Sporulation commenced when vegetative proliferation had ceased. The evidences obtained with sucrose as the carbon source for energy indicated that growth continued until the energy source was available in plenty. When it diminished to a level below the minimal requirements for the vegetative growth, morphological changes associated with sporulation started appearing and growth ceased.

Various stages in the transformation of a vegetative cell to a spore cell have been described. Very briefly, the sequence of events in this process started with the appearance of granules in the cytoplasm of the vegetative cell. This premonitory sign was followed by an increase in the length of the cell and later by the appearance of a club-shaped form at one end of the cell. The club-shaped portion of the cell later slowly became refractile to form the forespore, an evident sign of spore being formed. The forespore upon condensation in size, formed the sporangium, a stage almost of formed spore, but immature and still attached to its vegetative counterpart. However, the sporangium was soon released from the vegetative part and it then ripened to form the oval to round, fully refractile endospore.

In experiments where very low concentrations of energy source was used, it was observed that 100% spores were not formed and the cells that failed to form spores were being lysed. From this observation it was presumed that a basal level of energy source was necessary for the maintenance of structure and physiological integrity. This also indicated that energy source was required not for the growth only but for sporulation too.

With the aid of six electron micrographs, an attempt was made to correlate the events that followed the "triggering" for sporulation in various experiments.

Possible approaches and avenues for future investigations as well as the scope for the applications of the techniques developed were suggested and described. It is believed that the results obtained regarding the metamorphosis of the vegetative cell to spore, the effects of energy source sucrose, as well as the techniques developed, quite befittingly dovetail with the material in the literature related to the problem of sporogenesis.